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13. Abstract ***(Maximum 200 Words)*** (abstract should contain no proprietary or confidential information)
Nearly 20% of men who undergo a radical prostatectomy later relapse with bone metastases. The cellular events that are predictive of subsequent progressive disease remain unknown. We've focused attention on the detection of disseminated prostate cancer (CaP) cells in the blood and bone marrow. Our hypothesis is that these disseminated cells may provide critical insight regarding biomarkers of use in prognostication. We've developed enrichment and isolation techniques that allow the isolation of individual disseminated CaP cells for study as a pool of cells or single cells. Our proposal is to isolate these cells from 50 patients prior to radical prostatectomy and from 10 patients with advanced disease. The cells will be both phenotypically and molecularly analyzed. To date 96 patients have been accrued and the analyses well underway. For example, we are finding that ~57% of patients prior to radical prostatectomy have disseminated CaP cells in their bone marrow. Also, we show a correlation between patients with cytokeratin positive cells and human epithelial positive cells. In 56% of specimens having disseminated CaP cells we found aberrant features of chromosome 8 by FISH analysis. Micro-array gene expression analysis is planned on single cells from the first 10 patients to recur.

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Table of Contents

INTRODUCTION

One of the great challenges in the clinical management of prostate cancer (CaP) is determining the risk of progression in those patients thought to have localized disease. The recurrence rate among men who elect a radical prostatectomy for presumed localized disease ranges from 15 to 25%. Historically, two approaches have been taken to detect disseminated cancer cells and to improve the "staging" of patients at the time of diagnosis. In the first, microscopic scrutiny of disseminated cells in the blood of cancer patients by immunohistochemistry (IHC) and cytogenetic techniques revealed important information regarding the features of these disseminated cells in general (1-7). On a parallel path were efforts employing molecular technology. For example, ten years ago we (8) and Moreno (9) were the first to propose using the molecular technique of reverse-transcriptase-polymerase-chain-reaction (RT-PCR) to "molecularly stage" patients at diagnosis in hopes that detection of disseminated PSA+ cells in blood or bone marrow (BM) would be predictive of recurrence. Over time, efforts in this field showed that PSA RT-PCR positivity was not highly correlative with recurrence. However, we postulated that enrichment of the presumed CaP cells in the BM aspirate prior to RT-PCR testing might reveal a high pre-surgical detection rate and that techniques might then be developed to isolate the individual disseminated cells for study. We made use of magnetic particle cell enrichment techniques to study disseminated cells in CaP patients (10, 11, 16, 18). This approach provides for a much more robust analysis of the disseminated cell population, especially since the yield from BM aspirates can be several hundred to thousands of cells of interest. One of the most striking revelations in our series has been the documentation that ~55% of CaP patients prior to radical prostatectomy have disseminated cells in their BM aspirates following enrichment. The Objectives of this proposal center upon the study of these disseminated cells recovered from blood and BM and whether their biological features overall or as individual cells are predictors for progression. It appears logical that residual disseminated CaP cells in the BM (~90% of patients with advanced disease have bone metastases (12)) following a radical prostatectomy should provide at least as much insight as those associated with the primary tumor that is removed at surgery. The enriched population is adequate for microscopic analysis or for isolation of individual cells for further study. A powerful component of our proposed analyses involves cDNA micro-array-gene chips developed by our colleagues Leroy Hood and Peter Nelson. This technique combines the proven chemistry of nucleic acid hybridization with advanced automation and image analysis to quantitatively assess gene expression profiles (13-15). The expression levels of thousands to tens of thousands of genes represented at 0.01-0.001% abundance in a population can be simultaneously assessed. Thus our ongoing studies focus on generating molecular and IHC phenotypes of disseminated BM cells in fifty patients who are undergoing radical prostatectomy and having a Gleason Sum of 7 or greater. These profiles are contrasted to those of the primary tumor and to those in patients who have relapsed with bone metastases. A unique aspect of this proposal is our profiling of not only pools of disseminated cells but individual cells as the technologies advance to this level of sensitivity. Furthermore, comparisons of profiles will be made between these patient populations and a subset that demonstrates PSA biochemical relapse. These studies will provide the first multiparameter phenotypic analysis/discovery of potential progression markers that takes advantage of advances (a) in the recovery of disseminated CaP cells from blood and bone marrow, (b) in technology allowing the isolation of viable CaP cells to provide three degrees of heterogeneity (enriched, pooled homogeneous and individual cells) and (c) in the fields of cDNA microarrays and informatics that target small cell numbers

BODY

Hypothesis:

Disseminated CaP cells isolated from the blood and BM at the time of radical prostatectomy will reveal biological features useful in assessing the probability of relapse. Furthermore, the analysis of single cells isolated from the enriched population will provide a second level of discrimination reflective of the heterogeneity of disseminated cells and allow detection of rare, but important, features not revealed in pooled, groups of disseminated cells.

Technical Objectives:

Task #1: Define the predominant phenotype(s) of prostate cancer bone metastases

- Identify 10 patients with advanced prostate cancer involving the bone who have had a radical prostatectomy and were found to have a Gleason Sum $\geq$ 7. Following informed consent, obtain bone marrow aspirates. Using our para-magnetic enrichment techniques, we will derive an enriched population of disseminated CaP cells. From this population, we will "pluck" individual CaP cells and pool into sets of 50-100. These are then phenotyped using RT-PCR, PCR, FISH, ISH and micro-arrays (micro-array core facility of Peter Nelson, proposal co-investigator).
  (0 – 12 months)

Task #2: Establish phenotype(s) of primary tumor, and disseminated cells in blood and bone marrow by enrichment and pooling of cells from 50 patients undergoing prostatectomy and having Gleason Sum $\geq$7.

- Under separate funding we routinely obtain blood and bone marrow aspirates from all consenting patients prior to radical prostatetomy. From this large population this proposal involves a subset that consists of patients who are found to have a Gleason Sum $\geq$ 7. We will select 50 of these patients for the studies herein. Using our para-magnetic enrichment and isolation techniques, we will derive populations of disseminated CaP cells in three degrees of "purity": (a) enriched, (b) isolated individual cells that are pooled (50-100 cells) and (c) individual cells (20+). Using the phenotyping protocols of Task #1, sets (a) and (b) will be phenotyped from blood and from bone marrow. Set (c) will be partially purified and stored at -80° C for use in Task #3. We will also obtain at least 2 primary tumor foci (microdissected) for phenotyping.
  (0 – 24 months)

Task #3: Establish the phenotype of individual disseminated cells following enrichment and isolation from the bone marrow in patients of Aim #2 who experience biochemical (PSA) failure.

- Among the cohort of 50 patients, monitor for PSA biochemical recurrence using our ultrasensitive PSA chemiluminescent assay with which we've reported confidence in detecting relapse at a PSA serum level of 0.05 ng/mL.
  (0-30 months)

- Identify the first 10 patients who experience a biochemical recurrence and 10 who do not show any evidence of recurrence. Retrieve from –80°C storage the individual aliquots of disseminated CaP cells (bone marrow derived only) that were processed in Task #2. Phenotype, primarily by micro-array, these 400 individual CaP cells (20 cells/patient x 20 patients [10 fail + 10 no fail]). (20 – 36 months)

- Compare and contrast all data sets, perform statistical analyses. (30 – 36 months)

Results:

We have obtained a one-year no cost extension to complete the studies proposed. Therefore this is a progress report and not the final report.

As in the past, this year saw exceptional progress in some areas and slower progress in others. We were delayed by nine months in year 1, in our plan to accrue patients into the study because of a disagreement between the University of Washington IRB and the DoD over language in the consent form. A total of 60 patients were to be accrued, consisting of two patient populations, prior to radical prostatectomy and advanced disease. I am very pleased to reveal that we've far exceeded our accrual goals of 50 patients in the pre-radical prostatectomy patient group by now having accrued 94 patients. However, we still need to accrue 8 patients with advanced disease.

I mentioned in last year's report that with non-DoD funding, we acquired a better fluorescent microscope with which we achieved more accuracy in the detection of the disseminated cancer cells and the performance of fluorescent dependent phenotypic assays. I noted that there became a distinction among two populations of enriched epithelial cells from the bone marrow aspirates which had not been noticed with the older microscope. Our procedure uses negative (CD45 and CD61) selection followed by positive selection (anti-human epithelial antigen; HEA). The positively selected epithelial cells are detected under fluorescence using a FITC-labeled anti-HEA antibody to a different epitope than the antibody used for selection. The detected cells are then retrieved (i.e. "plucked") using a micromanipulator and pipette system attached to the inverted fluorescent microscope. In addition to the vast majority of non-fluorescent cells we noted now the two populations of fluorescent cells. One population was quite a bit more lightly stained than the other and in excess. The brightly stained population appeared to be the disseminated prostate cancer cells by morphology and molecular analysis whereas the lightly stained population appeared to be cells of other non-epithelial origin. An extensive literature search revealed one paper by Lammers et al (19) which stated that the human epithelial antigen resides in low abundance on a pre-erythroid stem cell population. Follow-up phone calls to other investigators using anti-HEA and to the commercial sources of anti-HEA antibodies failed to derive supportive evidence of this work. However, because the selective "plucking" of these cells followed by RT-PCR failed frequently to reveal a PSA message, we decided to retrieve only the more brightly stained cells for study. The consequence of this decision was that far fewer cells were obtained than with the other older microscope which did not distinguish among the two populations. Because of this, we have been forced to reduce the number of descriptive phenotypic studies in some patient samples in preference to storing the most positive cells for micro-array analysis which is to commence shortly. This has especially impacted the peripheral blood specimens where we have preferentially stored the few positive cells per sample for future single cell micro-array studies rather than attempt phenotypic studies on just a very few cells. The percentage of patients prior to radical prostatectomy with disseminated cells in these 94 pre-radical prostatectomy patients is currently ~33 % in the peripheral blood and ~57% in the bone marrow. Pools of positive cells and sets of individual positive cells have been obtained on all patients accrued to date where there were a sufficient number of disseminated cells detected. The radical prostatectomy patients are being monitored for biochemical failure as part of Task 3. At two years follow-up we have two failures.

On all of the patients where we acquired a peripheral blood specimen and bone marrow aspirate prior to surgery we also obtained a tumor specimen at radical prostatectomy. These tumor specimens were embedded in OCT and flash frozen. As part of Task 2, two foci from each specimen in patients yielding a sufficient number of disseminated CaP cells for analysis, are to be microdissected for comparison to the results from the disseminated cells. Due to difficulty in scheduling sufficient time for microdissection on a core laser capture microdissection system, we acquired non-DoD funding to purchase a system for ourselves. This system has been installed and we are on schedule to have all of the matched tumor foci microdissected within the next 30 days. As noted in the next paragraph, we are with-holding microarray analysis on these specimens until a reliable method is derived for the small cell number micro-arrays so that the identical method is used on both specimen types.

The ability to perform micro-array analysis on less than several hundred cells, let alone pools of approximately 20 cells or single cells as we proposed, has been an excrutiating technical challenge to those in the field. We had hoped to report that this challenge has been resolved and that analysis of the stored disseminated cells from our patients had begun. While this is not yet the case, we are pleased that significant but slow progress is being made. Pete Nelson, M.D. who is our collaborator at the FHCRC and his team have worked on this challenge. For example, we provided sets of LNCaP cells containing from 10 to 2500 cells to test the feasibility of two rounds of linear amplification followed by quantitative RT-PCR analysis. I am showing Figures

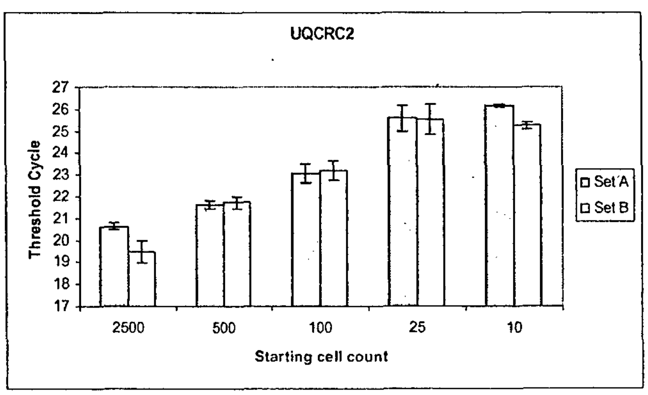

Figure 1

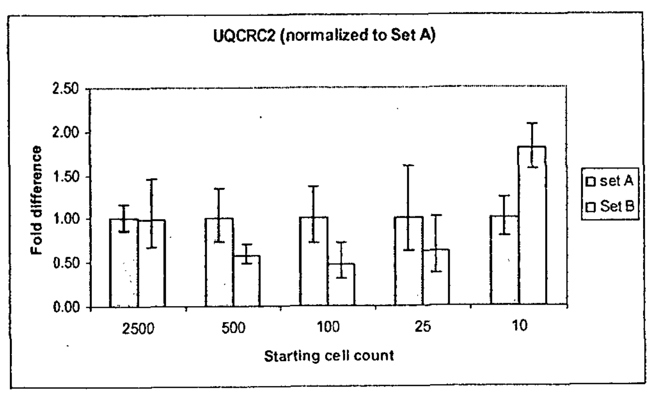

Figure 2

1 and 2 from last year's report again to highlight this issue. Up to 70 ug of aRNA was obtained from 10 LNCaP cells. Figures 1 and 2 demonstrate the success of these studies. In Figure 1, we demonstrate that the threshold cycle increases as the cell count decreases per unit aRNA reproducibly between sets; the difference between the samples with highest and lowest cell number is only 5-6 cycles. Figure 2 shows that throughout this range the fold difference is quite reproducible among samples, differing by less than 2 fold. While it is still conceivable that we will not be able to achieve the technical sensitivity to analyze the isolated single cells, analysis of the small pools of individual cells seems highly likely in the near term.

Progress has also been made in the phenotypic analysis of the disseminated cell population from the BM aspirates. Using a cytokeratin stain, we obtained percentages of patients positive for cytokeratin positivity (75%) and HEA positivity (57%) in BM. In the study of double-staining for cytokeratin and Ki-67, only 11% (8/74) of the patients with cytokeratin positive cells also revealed Ki-67 stained cells. Within this small population, the percentage of cytokeratin positive cells that were Ki-67 positive was generally <10%. Thus, very few of the disseminated prostate cancer cells in the pre-radical prostatectomy population are actively undergoing division. A rare example of a Ki-67 positive cell (also cytokeratin positive) obtained from a bone marrow aspirate is shown as Figure 3, (also shown last year).

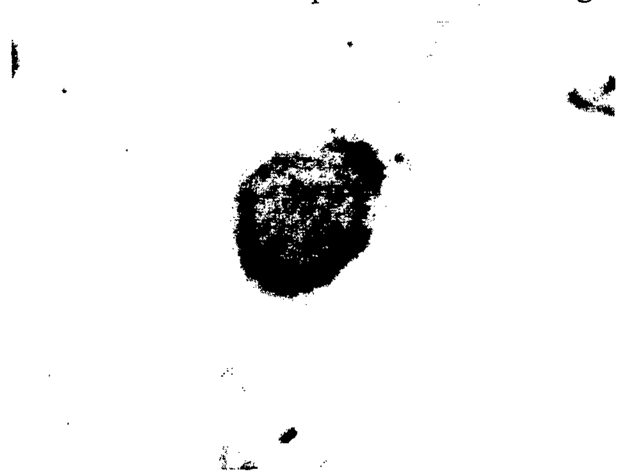
Figure 3

One interesting notation that conforms to our previous observations is that approximately a third of the patients with HEA positive and/or cytokeratin positive cells do not have PSA staining cells. This implies that these cells, once separated from the supportive stroma, stop producing PSA at levels necessary for immunohistochemistry detection.

Nearly all of the bone marrow specimens having cytokeratin positive cells (N=70), have been analyzed by FISH (Vysis ProVysion). This multiprobe kit for chromosome 8 allows for detection of increase or decrease of chromosome 8 and deletion/loss of 8p/8q. We had originally analyzed all specimens regardless of the number of cytokeratin positive cells (N=52 specimens). However, it soon became apparent that those with a low number of cytokeratin cells were not very reliable. Therefore, for final analysis we are only evaluating those with 10 or more cytokeratin positive cells. The detailed analysis is underway but in 22 of 39 specimens (56%), chromosome 8 aberrations were noted. This is further evidence that malignant cells comprise at least a segment of the disseminated cell population. Figure 4 shows a disseminated, cytokeratin positive cell (A) with 2 copies of chromosome 8 and 8q with one copy (red) of 8p (B).

Figure 4A

Figure 4B

For RT-PCR analysis, we had originally planned on assessing Prostate Stem Cell Antigen (PSCA) and Prostate Specific Membrane Antigen (PSMA) on the enriched cell population but after dozens of analyses, concluded that there was too much background from the contaminating white blood cells to make an accurate determination of whether the signal was indeed from disseminated prostate cancer cells. Therefore, we attempted to do these analyses on ~10 – 20 of the individually "plucked" and then pooled cells from the enriched bone marrow aspirates. Using a pool of 10-20 LNCaP cells we can consistently obtain a positive PSMA RT-PCR result. We are now in the process of converting to a real-time RT-PCR assay. Ideally, this may be the preferred assay format. We are very cautious about using the actual 10-20 disseminated cell pools because they are irreplaceable. However, we expect to make a decision on the analysis method in the very near future. In regard to PSCA, we are not as optimistic. Using a 10-20 cell pool we have been unable to consistently obtain the sensitivity and specificity required. Thus, at this point, we do not believe we will be able to include PSCA RT-PCR in the analysis. In last year's report, we discussed the possibility of using EZH2 (17) RT-PCR instead of PSCA RT-PCR. Here we encountered a different problem. Although we could get the sensitivity down to 10-20 cells routinely and one CaP cell frequently, we consistently obtained a positive EZH2 RT-PCR result on white blood cells. Thus, it is not a reliable specific marker for CaP cells even in the pooled disseminated cell population, since a few contaminating cells among the 20 "plucked" cells could give a false positive result. These difficulties highlight the significant technical hurdles being encountered in the accurate phenotypic and molecular assessment of individual and extremely small cell populations. It is becoming more accepted that even with the one year no-cost extension, some of the studies originally proposed will not be completed due to insurmountable technical hurdles. Nevertheless, considerable effort is being expended to solve these problems.

KEY RESEARCH ACCOMPLISHMENTS

- Following a very slow start of patient accrual in year 1 due to IRB administrative issues, we have now exceeded our goal of 60 patients by enrolling 96. Due to low disseminated cell numbers in some of these pre-prostatectomy patients we increased the accrual target. We received a no-cost, one year extension to allow time for biochemical recurrences to occur in 10 patients and to devote additional effort in solving the technical hurdles in molecularly phenotyping individual and small sets (10-20 cells) of cells..

- We've made extensive progress in the phenotypic analysis of the disseminated cells from the bone marrow aspirates using the markers PSA, cytokeratin and Ki-67 and in FISH analysis of chromosome 8. However, the GSTpi studies have not given reproducible results at single cell and extremely low cell numbers.

- Good progress has been made in assessing specific gene expression profiles of isolated pools of 20 individual cells by RT-PCR or real-time RT-PCR including PSA, PSMA, and EGP. AR methylation studies have begun only in those patients where sufficient disseminated cells are available as it is of lower priority. At this time, it appears that AR methylation studies will be performed in a minority of the patient specimens due to insufficient cell numbers. Our efforts to analyze PSCA on very few cells has nearly reached an impasse, i.e. we cannot achieve the sensitivity and specificity required to analyze 10-20 cells or individual cells. This again highlights the technical hurdles being encountered as we push the limits of molecular phenotyping down to single cells or pools of 10-20 cells. With clinical specimens of individual and pooled cells so precious, we can not afford to engage in analysis of these specimens unless we are confident that the results are accurate.

- During the past few months, with non-DoD funding we acquired our own laser capture microdissection system. This has allowed a much more rapid dissection of tumor foci from the primary tumor removed at radical prostatectomy. In fact, within the next 30 days we should be up to date on these resections. Although there are sufficient cells in these dissections to do cDNA micro-arrays, it is critical that the identical procedure be used when comparing the microdissected tumor foci to the disseminated cells. However, as noted below, we have not as yet finalized the method of amplification and analysis by micro-arrays of single cells or small pools (i.e. 10-20 cells) of the disseminated cells. Therefore, our tumor foci dissections are now simply being stored until that procedure is finalized.

- Despite intensive efforts, we are still not comfortable with gene expression microarray analysis using a single cell or even a pool of 10-20 individual cells. These pools of single disseminated cells and the individual cells have been "plucked" and stored from the patient samples but because they are so valuable, we won't engage in the microarray analysis until the procedure is more robust. This is a huge technical challenge, as are many of the aspects of this proposal. Slow but steady progress is being made by our collaborator Peter Nelson in reaching the goal of using few to single cells for this task.

REPORTABLE OUTCOMES

Abstracts (cumulative):

Pfitzenmaier J, Arfman E, Klein J, Winch R, Nance M, Lange P and Vessella RL. New enrichment method for the isolation and characterization of circulating prostate cancer cells (CPCC) from the peripheral blood (PB). Proc. Am Assoc. Cancer Res 43:433 #3635, 2002

Pfitzenmaier J, Ellis WJ, Arfman E, Klein JR, Lange PH and Vessella RL. A method to isolate disseminated prostate epithelial cells and the comparison of their detection rate to standard RT-PCR. Proc. Am Assoc. Cancer Res 44:42, #216, 2003.

Pfitzenmaier J, Ellis WJ, Arfman EW, McLaughlin PO, Lange PH and Vessella RL. Telomerase activity in circulating prostate cancer cells. Proc. Am Assoc. Cancer Res 44:42, #5193, 2003

Lin, DW, Pfitzenmaier J, Ellis WJ, Arfman EW, Klein JR, Lange PH and Vessella RL. Detection and isolation of PSA positive epithelial cells by enrichment: comparison to standard PSA RT-PCR, clinical relevance and initial characterization in prostate cancer patients. American Urological Association Annual Meeting, Abstract #837, 2004.

CONCLUSIONS

Our year 3 progress, especially in patient accrual, is a great contrast to the slow start in year 1 due to the nine month delay in patient accrual due to DoD IRB issues. We have exceeded our pre-radical prostatectomy goals by nearly 100% but will continue to accrue these patients as less than a third have yielded a sufficient number of disseminated prostate cancer cells to perform all of the studies proposed. We have processed all of the peripheral blood and bone marrow aspirates, enriching for disseminated prostate cancer cells. On those specimens with evidence of disseminated prostate cancer cells we have made suitable progress in both the phenotypic and molecular characterization of these cells. Also aliquots of individual cells have been "plucked" and stored until there is evidence of biochemical recurrence in at least 10 radical prostatectomy patients (mean followup period is only 1 year at present). While several technical challenges remain ahead of us, significant progress is being made. To our knowledge, we still remain the only group applying the most advanced micro-array expression technologies with those of classical phenotyping and FISH analysis to the study of disseminated prostate cancer cells isolated from the bone marrow down to the single cell level. While the technical challenges must not be underestimated and some goals may not be achieved because of this, the opportunities for advancement of knowledge remain highly attractive. The granted one year no-cost extension will provide additional time to hopefully resolve some of these technical hurdles and allow time for detection of biochemical recurrences.

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